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Abstract

The analysis of the global thiol-disulfide redox status in tissues and cells is a challenging task since thiols and disulfides can undergo artificial oxido-reductions during sample manipulation. Because of this, the measured values, in particular for disulfides, can have a significant bias. Whereas this methodological problem has already been addressed in samples of red blood cells and solid tissues, a reliable method to measure thiols and disulfides in cell cultures has not been previously reported.

Here, we demonstrate that the major artifact occurring during thiol and disulfide analysis in cultured cells is represented by glutathione disulfide (GSSG) and *S*-glutathionylated proteins (PSSG) overestimation, due to artificial oxidation of glutathione (GSH) during sample manipulation, and that this methodological problem can be solved by the addition of N-ethylmaleimide (NEM) immediately after culture medium removal. Basal levels of GSSG and PSSG in different lines of cultured cells were 3-5 and 10-20 folds higher, respectively, when the cells were processed without NEM. NEM pre-treatment also prevented the artificial reduction of disulfides that occurs during the pre-analytical phase when cells are exposed to an oxidant stimulus. In fact, in the absence of NEM, after medium removal, GSH, GSSG and PSSG levels restored their initial values within 15-30 min, due to the activity of reductases and the lack of the oxidant. The newly developed protocol was used to measure the thiol-disulfide redox status in 16 different line cells routinely used for biomedical research both under basal conditions and after treatment with disulfiram, a thiol-specific oxidant (0-200 μM concentration range).

Our data indicate that, in most cell lines, treatment with disulfiram affected the levels of GSH and GSSG only at the highest concentration. On the other hand, PSSG levels increased significantly also at the lower concentrations of the drug, and the rise was remarkable (from 100 to 1000 folds at 200 μM concentration) and dose-dependent for almost all the cell lines. These data support the suitability of the analysis of PSSG in cultured cells as a biomarker of oxidative stress.

Keywords: glutathione, glutathione disulfide, *S*-glutathionylated proteins, cell cultures

Abbreviations

actinSSG, S-glutathionylated actin

BAEC, bovine aortic endothelial cells

BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea

DDC, diethyldithiocarbamate

DMEM, Dulbecco's modified eagle medium

DTT, dithiothreitol

EMEM, Eagle's minimum essential medium

FBS, fetal bovine serum

GR, glutathione disulfide reductase

GSH, glutathione

GSSG, glutathione disulfide

HUVEC, human umbilical vein endothelial cells

mBrB, monobromobimane

NEM, N-ethylmaleimide

PBS, phosphate buffered saline solution

PSSG, *S*-glutathionylated proteins

R-SH, thiols

ROS, reactive oxygen species

TCA, trichloroacetic acid

TNF-α, tumor necrosis factor α

Introduction

Thiols (R-SH) have been and continue to be of interest because of their important role in a number of biological processes. The most peculiar properties of the sulfhydryl (-SH) group, which determine both its chemistry and biology, are the ready oxidizability and fast reactivity, unlike disulfides that are much less reactive than thiols. Most -SH groups are contained in protein cysteinyl residues, whereas almost all non-protein -SH is represented by glutathione (GSH). GSH plays a key role in cell resistance to oxidative and nitrosative damage by providing reducing equivalents for enzymes involved in the metabolism of reactive oxygen/nitrogen species, as well as by eliminating potentially toxic oxidation products and reducing oxidized or *S*-nitrosated proteins. In addition, it contributes to the metabolism of exogenous molecules in phase II detoxification reaction by its addition to electrophilic centers of xenobiotics [1]. Within mammalian cells, glutathione exists mainly (>98%) in the thiol-reduced form (GSH), whereas glutathione disulfide (GSSG) is usually <1% of GSH [2]. The GSH/GSSG ratio is normally closely regulated. GSSG is reduced to GSH by the enzyme glutathione disulfide reductase (GR) in a reaction requiring NADPH as a cofactor, or is exported from cells when its intracellular concentration increases [3,4]. GSH/GSSG ratio is a critical mechanism for cell survival; in fact, it is known that it varies in association with proliferation, differentiation, and apoptosis [5]. Under severe oxidative stress, this ratio can drastically shift. It has been established that decrease in GSH, increase in GSSG levels, and decrease in the GSH/GSSG ratio, are important contributing factors to some human diseases, including diabetes mellitus, cardiovascular diseases, neurodegenerative diseases, and cancer [6-8]. Furthermore, cellular GSH/GSSG redox state seems to represent an important component of malignant cell survival [9].

Changes in GSH/GSSG ratio can potentially affect a number of target proteins by promoting oxidation and/or disulfide exchange reactions at specific protein cysteine residues [2]. Protein *S*-glutathionylation (PSSG) is a post-translational modification that takes place by thiol-disulfide

exchange between protein –SH groups and GSSG. This reaction can effect a change in conformation and/or charge that may alter protein function. In fact, by adding glutathione tripeptide to a target protein, an additional negative charge is introduced (as a consequence of the Glu residue), and a modification in protein conformation is made [10]. *S*-glutathionylation is attracting growing interest both as biomarker of oxidative stress and as a biological switch involved in a number of critical oxidative signaling events by modulation of the activity of several enzymes (e.g., kinases and phosphatases), transcription factors, and oncogenes [11,12].

Because GSH and its disulfide forms are involved in many essential cellular functions, reliable precise and accurate methods to identify and quantify them are in high demand. The analysis of the global thiol-disulfide redox status in tissues and cells is a challenging task for three main reasons: 1) thiols easily undergo auto-oxidation during the pre-analytical phase of sample preparation; 2) disulfides can be reduced back to thiols enzymatically during cell manipulations; 3) basal levels of disulfides (GSSG and PSSG) are very low [13,14]. Consequently, without particular care to sample manipulation, disulfide levels might be overestimated or underestimated, altering the normal thiol/disulfide ratio and, consequently, making the interpretation of results erroneous. Our previous investigations carried out in blood components and solid tissues indicate that artificial alteration of the thiol/disulfide balance can be prevented by as rapid-as-possible thiol alkylating and GR inhibition. The use of N-ethylmaleimide (NEM) was shown to be the most effective preventing agent [13, 15-17]. Correct analysis of thiol/disulfide balance may be more complex in cell cultures than in other biological systems because many different experimental conditions can be used to treat and collect them, thus introducing many interfering factors. To our knowledge, a reliable method to measure GSH, GSSG and PSSG in such samples has not yet been reported.

Here, we have developed and validated a new protocol to measure GSH, GSSG and PSSG in a large series of cell lines. Results indicate that GSSG and PSSG values reported in the literature are markedly biased by incorrect sample collection and manipulation, which alter thiol/disulfide levels. In addition, we studied thiol/disulfide homeostasis in cells after treatment with disulfiram, a

drug clinically used to support the treatment of chronic alcoholism [18]. Disulfiram possesses a reactive disulfide bond, which can react with both protein and low-molecular mass thiols forming mixed disulfides, disulfides, and dithiocarbamates. Therefore, it is a useful model to study how the thiol to disulfide balance is modified in response to an oxidant stimulus.

Material and Methods

Cell cultures

Bovine aortic endothelial cells (BAEC) were obtained from bovine thoracic aorta by enzymatic digestion with 0.05% (w/v) type-2 collagenase (Worthington, Lakewood, NJ) and grown in Dulbecco's modified eagle medium (DMEM, Sigma Aldrich, Milan, Italy) supplemented with 20% fetal bovine serum (FBS, Euroclone, Milan, Italy), 100 μg/ml endothelial cell growth supplement (Sigma Aldrich, Milan, Italy), 100 U penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Sigma Aldrich, Milan, Italy). Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cords collected from uncomplicated pregnancies and grown on 1% (w/v) gelatin (Sigma Aldrich) in EBM-2 medium (Lonza). A549 (human lung carcinoma-derived cells), NT2-D1 (human pluripotent embryonal carcinoma-derived cells), RD (human rhabdomyosarcoma-derived cells), IMR90 (human embryonic lung-derived fibroblasts), HEK293 (human embryonic kidney-derived cells), A375 (human melanoma-derived cells), Panc-1 (human pancreatic carcinoma-derived cells), and T98G (human brain glioblastoma-derived cells) were obtained from ATCC® (American Type Culture Collection Manassas, VA). HaCaT (human spontaneously immortalized keratinocyte cells) were obtained from Cell Line Service (Eppelheim, Germany). All these cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U penicillin, 100 μg/ml streptomycin and 2 mM glutamine. MCF-7 (human breast cancer-derived cells, from ATCC®) and BRC-230 (human breast cancer-derived cells, isolated by Amadori et al. [19]) were maintained in DMEM/Ham's F12 (1:1) supplemented with 10% FBS, 2 mM glutamine, and insulin (10 μg /ml; Sigma Aldrich). LNCaP (human prostate cancer-derived cells, from

ATCC®) were grown in RPMI (Life Technologies Ltd) supplemented with 10% FBS and 2 mM glutamine. LNCaP-Rbic (bicalutamide-resistant cells, derived from LNCaP and isolated by Pignatta et al. [20]), was maintained in the same medium of parental cells supplemented with 20 μM of (R)-bicalutamide (Casodex®). U87 (human glioblastoma-derived cells, from ATCC®) were grown in Eagle's Minimum Essential Medium (EMEM, ATCC®) supplemented with 10% FBS.

All cells were cultured in an atmosphere of 95% air and 5% CO2 at 37°C in 100 mm culture plates. All the cell lines used in the present study were checked periodically for mycoplasma contamination by MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Identification of artifacts for thiol and disulfide analysis and validation of the protocol in BAEC

At confluence, the culture medium was removed and cells were washed twice (1 min each) with phosphate buffered saline solution pH 7.4 (PBS) containing 5 mM NEM (Sigma Aldrich, Milan, Italy). In comparative experiments, NEM was omitted in phosphate buffered saline and added successively for HPLC analysis. Cells were then lysed by treatment with 0.5 ml of 4 % (w/v) trichloroacetic acid (TCA, Sigma Aldrich, Milan, Italy) and collected after scraping. Samples were either immediately analyzed or stored at -80°C until analyses. Measurements were always carried out within 10 days from sample preparation. Previous experiments had revealed that samples were stable at least for 1 month at -80°C.

In experiments with pro-oxidant treatment, cells at confluence were treated with 0.2 mM hydrogen peroxide (Sigma Aldrich, Milan, Italy) for 10 minutes. The culture medium was then removed and cells were washed twice with PBS containing or not NEM as described above. In some experiments, 0.1 mM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was added to the washing buffer without NEM, in order to study the effect of GR on GSSG reduction during sample handling [21].

For the recovery study, GSH, GSSG, and S-glutathionylated actin (actinSSG) were added at three concentrations to the cells immediately before the lysis with TCA. Cells were then processed

as described above.

Measurement of intracellular GR activity

After BCNU treatment, BAEC were collected by tripsynization, sonicated, followed by centrifugation at 14,000g for 10 min at 4 °C, and the supernatant collected and stored at -80°C. GR activity was measured by monitoring the NADPH oxidation by spectrophotometer [22,23].

Cell treatment and preparation for thiol and disulfide analysis

Cultured cells were used at 90% of confluence. All cell lines were treated with disulfiram (Sigma Aldrich, Milan, Italy, freshly dissolved in dimethyl sulfoxide and diluted in the culture medium) in the concentration range of 0-200 μM for 15 minutes. The culture medium was then removed and cells were washed twice (1 min each) with PBS containing 5 mM NEM. Cells were then processed as described above. Measurements were always carried out within 10 days from sample preparation.

Preparation of actinSSG

ActinSSG was prepared according to Dalle-Donne et al. [24]. Briefly, 30 μM G-actin in 2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM NaN_3 was treated with 5 mM diamide (Sigma Aldrich, Milan) for 30 min and then with 5 mM GSH for 1h at room temperature. Sample was then acidified with 6% (w/v, final concentration) TCA and the protein pellet was titrated for the PSSG content by HPLC, as described below, and stored at -80°C.

Measurement of GSH, GSSG, and PSSG in cells

Cells in TCA were centrifuged at 10,000xg for 2 minutes. GSH was measured on the clear supernatant as the GS-NEM conjugate by UV-Vis HPLC [25]. In samples where NEM was omitted

during the pre-analytical phase (see above), it was added after the acidification and centrifugation steps together with 10 µl of 2 M Tris base. Samples were incubated for 5 minutes at room temperature and then acidified by addition of 2 µl of 37% (v/v) HCl. GSSG and PSSG were measured by quantifying by HPLC the GSH released by the cleavage of the disulfide bridge by dithiothreitol (DTT, Sigma Aldrich, Milan, Italy) and labeled with the fluorescent probe monobromobimane (mBrB, Calbiochem) [26]. Briefly, for GSSG analysis, one aliquot of supernatant was reacted with 3 volumes of dichloromethane for 10 minutes on a rotator shaker in order to remove the excess of NEM. One hundred microliters of supernatant were then treated with 4.2 µl of 5 mM DTT in the presence of 20 µl of 2 M Tris base. After a 20-minute incubation at room temperature, samples were deproteinized by addition of 20 µl of 60% (w/v) TCA and centrifugation at 10,000xg for 2 minutes. One hundred microliters of supernatant were then reacted with 3 µl of 40 mM mBrB in the presence of 20 µl of 2 M Tris base for 10 minutes at room temperature in the dark. Finally, samples were acidified by addition of 5 µl of 37% (v/v) HCl and analyzed by HPLC. For PSSG analysis, protein pellet was washed twice with 1.5% (w/v) TCA and resuspended in 0.3 ml of 0.3 mM DTT in 1 mM K_3EDTA and 15 µl of 2 M Tris base. Samples were incubated for 20 minutes at room temperature on a rotator shaker and then deproteinized by addition of 30 µl of 60% (w/v) TCA and centrifugation at 10,000xg for 2 minutes. One hundred microliters of supernatant were then reacted with 4 µl of 40 mM MBrB and 20 µl of 2 M Tris base for 10 minutes in the dark. Finally, samples were acidified by addition of 5 µl of 37% (v/v) HCl and analyzed by HPLC.

Protein concentration determination

Determination of protein concentration was performed by the Bradford assay [27]. Protein acidified pellets were resuspended in 0.2 ml of 0.1 N NaOH. Bovine serum albumin was used as standard.

Statistics

Differences between means were evaluated using Student's t test. A value of $p < 0.05$ was considered statistically significant. Correlation analysis was performed by finding the best-fitting straight line through the data points, described by the general equation: $y = ax+b$, where a is the slope, b is the intercept with y axis and x and y are the two quantitative variables. The Pearson's product-moment correlation coefficient (r) was then calculated.

Results

Identification of artifacts and development of the protocol to stabilize thiols and disulfides within cells

We have previously demonstrated that an accurate measurement of thiols and disulfides in blood cells requires the masking of the –SH groups by NEM before sample acidification in order to protect thiols from an artefactual oxidation [13, 15-17]. Similar to blood cells, we hypothesized that also for reliably measuring thiols and disulfides in the cell lines routinely used for biomedical research this protection of thiols was necessary to avoid their possible artificial oxidation. In order to confirm this hypothesis, we measured thiols and disulfides in BAEC that were treated or not with a thiol alkylating agent (NEM) before their collection and lysis (Fig 1). When NEM was omitted GSH values did not vary significantly, whereas both GSSG and PSSG levels were one order of magnitude greater in respect to those measured in cell samples pre-treated with the alkylating agent just after medium removal. However, when the same cells were exposed to a pro-oxidant stimulus (0.2 mM H_2O_2 for 10 min) the levels of GSH were significantly higher and the levels of disulfides significantly lower in BAEC not treated with NEM with respect to the same samples pre-treated with the alkylating agent (Fig 2). We hypothesized that this effect was due to the activity of cellular reductases that work in the absence of the pro-oxidant stimulus during the time elapsing between washing phases and analytical measurements. In fact, in experiments where BCNU, a GR inhibitor, was added to the washing buffer, the oxidizing effect of H_2O_2 (i.e. decrease of GSH and increase of

GSSG and PSSG) was more evident with respect to the same samples without NEM (Fig. 2). Analysis of GR activity in BAEC treated with BCNU under the same experimental conditions showed a decrease of the activity by about 65%. Similar results on artificial oxidation/reduction in absence of NEM were obtained for other line cells tested, namely HUVEC, NT2-D1, RD, HEK239, A549, T98G (data not shown). We have also investigated the right concentration of NEM to be used. In experiments with BAEC we tested NEM at 0-5 mM concentration range in washing buffer. Our results indicate that 1 mM NEM was enough to bind all the free GSH occurring within cells (Fig 1S).

Measurement of GSH, GSSG and PSSG within cells: validation of the procedure and basal levels

In order to measure thiols and disulfides in different cell lines, a protocol that allows the simultaneous assay of GSH, GSSG, and PSSG in a unique sample was used. The protocol includes the fundamental step of -SH alkylation by NEM, then GSH is measured by the direct analysis of the GS-NEM conjugate by HPLC as recently described [25]. Instead, GSSG and PSSG detection (whose concentration in the intracellular compartments is generally 2-3 orders of magnitude lower that of GSH) requires fluorescent labeling of -SH groups with mBrB after reduction of the disulfide bridge [26]. The concentration of DTT, selected as reducing agent, needed an optimization, because of the low concentrations of both analytes (GSSG and PSSG), and the shortcoming of mBrB itself to generate some spurious HPLC peaks in the presence of reductants. To this aim, several concentrations of DTT were tested for the analysis of both GSSG and PSSG. As reported in Figure 2S, DTT concentrations corresponding to 0.1 mM (or higher) were analogously effective to induce release of all the GSH occurring as GSSG or PSSG, and to fit our needs. Robustness of our method was assess by measuring the recovery of standard solutions of GSH, GSSG and actinSSG added to pellets of BAEC at different concentrations. Recovery was always close to 100%, thus confirming method robustness (Table 1).

By using this method, we measured basal levels of GSH, GSSG, and PSSG in several cell

lines. Results are shown in Table 2. As expected, GSH was the prevailing form in all kinds of cells, with a ratio GSH to GSSG always >100. In some cell types (NT2-D1, HEK293, U87, and IMR90) the ratio was even higher (>500). In all the analyzed cells, PSSG concentration was lower than that of GSSG, with the exception of U87 and A375 cell lines (ratio GSSG/PSSG<1). It is worth noting that a strong correlation was observed between GSH/PSSG and GSSG/PSSG ratios (equation: $y = 2.76x + 0.313$; $r = 0.867$).

Effect of disulfiram

In cells treated with the thiol reactant disulfiram (concentration range 0-200 μM) a decrease in GSH levels was observed solely (with the exception of the A357 cell line) when elevated drug concentrations were used. This effect of disulfiram was not seen in all the analyzed cell lines. As shown in Figure 3A and Table 3, in Panc-1, NT2-D1, A549, HaCaT, BRC-230, MCF-7, LNCaP and LNCaP-Rbic cell lines, GSH levels were not affected by the drug. In contrast, disulfide forms (GSSG and PSSG) increased in all the studied cell lines (Figure 3B,C) and Table 3. However, GSSG increase was significant only at the highest dose of disulfiram in most of cell lines, with the exception of A375, HEK293, Panc-1, RD and IMR90 cell types, whereas the rise of PSSG was evident even at low doses of the drug. In most cases, the increase of PSSG was concentration-dependent and congruent with the decrease in GSH. Also, the ratio GSH/GSSG (Fig. 4A and Table 3) decreased by the treatment; however, with the exception of the HEK293 and A375 cell lines, this decrease was significant only at the highest concentrations of disulfiram (50 or 200 μM). Again, the oxidizing effect of disulfiram was largely more evident when ratios involving PSSG were calculated. The decrease of the GSH/PSSG ratio was significant at 10 μM concentration of the thiol reactant for all the studied cell lines with the exception of Panc-1, U87 and T98G (Fig. 4B and Table 3). The GSSG/PSSG ratio also decreased significantly at the lowest concentration disulfiram in most of analyzed line cells (Fig. 4C and Table 3).

Discussion

Cell cultures are widely used by scientific research, which includes the study of cellular response to oxidative stress, a condition characterized by an imbalance between the levels of oxidants (mainly reactive oxygen species, ROS) and antioxidants in favor of the former leading to potential damage [28]. Oxidative stress is supposed to be related to the onset and/or progression of many human diseases, and cell cultures represent an easy model (although with clear limits) to understand the underlying mechanisms and the potential instruments of defense.

Thiols are widely studied as targets of an oxidant insult, given their high reactivity and ubiquity in cells and tissues, and the GSH/GSSG ratio is frequently used as an indicator of the redox balance [14]. However, a key issue related to thiol and disulfide detection is represented by both artificial thiol oxidation and disulfide reduction during the pre-analytical phase. This problem has recently been addressed in red blood cells [15,16], in the other blood cells (this laboratory, unpublished results), and in tissues different from blood [13, 26].

Here, we show that an analogous artificial oxidation may occur when analyzing the cellular thiol/disulfide status in cell cultures, in particular during the acidification step. In fact, most methods currently used to measure GSH and its main oxidized forms require acid deproteinization of the sample to remove proteins before analysis. Unfortunately, all commonly used acids lead to a different extent of GSH oxidation, depending on various factors (kind of acid, its concentration, sample dilution and the type of biological matrix, oxygen concentration) [15,26,29]. The use of NEM, due to its property as an alkylating agent for thiols, prevents the artificial oxidation (Fig. 1). For this purpose, NEM is far more effective than other alkylating agents (such as iodoacetic acid, 2-vinylpiridine) for two main reasons: it rapidly crosses plasma membranes, and it reacts with thiols within a few seconds [14]. In NEM-treated cells, both GSSG and PSSG levels resulted to be significantly lower than those we measured within cells with free –SH groups, where an evident, artificial oxidation of GSH occurs. Our data indicate that about 1-3% of GSH is oxidized to GSSG and/or PSSG during deproteinization of cell lysates by acid treatment. Hence, it does not represent a

major bias in GSH assay. In contrast, artificial acidification becomes of dramatic importance for analysis of disulfides. In cultured cells, as well as in tissues, GSSG and PSSG occur at very low concentrations (0.2-0.6%, see Table 2) compared to GSH. Thus, a minimal oxidation of GSH may exert a great impact on GSSG and/or PSSG concentrations.

The use of NEM not only protects cellular GSH from the acid-induced oxidation, but also defends disulfides from the reduction that may occur during sample manipulation. The latter effect is particularly important when treatments with substances supposed to induce pro-oxidant stimuli are carried out. In fact, the pro-oxidant agent is generally removed from the medium before collecting cells. Cells are then processed in different ways (washing, trypsinization, scraping, centrifugation, and so on). During these pre-analytical steps, disulfide reduction may occur. Timely NEM addition allows to "freeze" the thiol to disulfide ratio exactly at that required time point, preventing uncontrolled artificial alterations. Our data (Fig. 2) indicate that if cells are processed without NEM, an underestimation of disulfide levels occurs, consequent to their reduction by the cellular enzymic machinery (e.g., GR, glucose 6-phosphate dehydrogenase and thioltransferase) in the absence of the oxidant, washed out from the culture medium. In fact, this phenomenon was reduced when a partial inhibition of GR activity was induced by treatment with BCNU. NEM prevents the underestimation of GSSG because, in addition to alkylate the entire cellular -SH groups, it is also a rapid and irreversible inhibitor of GR, by binding the Cys residue in the catalytic site [14, 15]. Moreover, GSH alkylation also inhibits the reduction of PSSG that may occur via thiol-disulfide exchange. Protein deglutathionylation (i.e., the detachment of glutathione from protein mixed disulfides) can also be catalyzed in mammalian cells by the thiol-disulfide oxidoreductase enzyme glutaredoxin (Grx, also known as thioltransferase) and, to a minor extent, by thioredoxin. Catalytic mechanisms are well known and essentially involve the cysteine residues in the active site of the enzymes and their attack to the glutathionyl sulfur of the protein–SSG disulfide bond [30-32]. Given that these cysteinyl residues are exposed and highly reactive, NEM

rapidly alkylate them, thus inhibiting any possible enzymatic reduction of PSSG, even if reducing equivalents from sources other than GSH (e.g., NADPH) can be used.

In this manuscript, we propose and validate a new procedure for accurate measurement of GSH and its oxidized forms (GSSG and PSSG) in cell cultures. It must be pointed out, in this context, that PSSG can be quantitatively detected by our method but, in order to obtain qualitative information (i.e. the specific protein targets of *S*-glutahionylation), alternative procedures are requested, such as the use of monoclonal antibodies or thiol-affinity matrices coupled to liquid chromatography-tandem mass spectrometry [33-35].

To the best of our knowledge, we report here, for the first time, the real unbiased levels of the thiol to disulfide ratio in different types of cultured cells. Our proposal consists in the addition of NEM immediately after culture medium removal. Even though 1 mM concentration seems to be sufficient to bind all GSH, we considered the use of 5 mM NEM to be safer, as different cell lines may have considerable intrinsic variability in GSH levels, and off-target effects of NEM are not foreseen. Once thiols and disulfides are stabilized by NEM, different procedures can be applied to detect them. GSH levels were in the same order of magnitude reported in the literature for the respective cell line [36-44]. No data exist for GSH in NT2-D1, U87, BRC-30 and LNCaP-Rbic cell lines. Instead, GSSG levels were at least one order of magnitude lower than those reported in the literature [37,40-42,44-46]. The important change was likely due to the artificial GSSG formation during sample processing that, in our experiments, was efficiently prevented by NEM. Consequently, the GSH/GSSG ratio in these cell lines was markedly higher compared with the levels reported previously. PSSG analysis is a quite demanding task given the low intracellular levels of this biomarker, especially under basal conditions (Table 2 and [47]). To our knowledge, this is the first time the basal levels are reported for the cell lines analyzed in this study.

The thiol to disulfide ratio across most of the studied cell lines was lower than the value found in red blood cells (about 500) [15,17]. The possibility that cell cultures *per se* undergo pro-oxidant conditions may explain these results. The experimental atmosphere used to grow cells (i.e.

high O_2 tension), the high levels of glucose, and antioxidant deficiency, common conditions in culture media, are all contributing factors for ROS generation, which, in turn, can influence many events of the cell life [48]. Therefore, it is possible that different cell lines, depending on selective metabolism pathway and specific adaptive strategies to culture conditions, increase their disulfide content. Preliminary data from our laboratory (experiments are still ongoing and data will be presented in future publications), aiming to evaluate the effect of a low O_2 tension (3%) on cellular thiol/disulfide balance, suggest that in many cases a low oxygen tension did not have a significant impact on thiol and disulfides levels (some of measured data are shown in Table 1, supplementary data). The only exception was represented by GSSG in NT2-D1, whose concentration resulted to be increased at 3% O_2. Interestingly, GSH levels also increased under these experimental conditions in NT2-D1, whereas they decreased in T98G cells, suggesting that cells can undergo some specific adaptive strategies involving GSH synthesis and/or uptake of the substrates, whose regulations are not yet completely understood. These differences underline the diversity in cellular responses to physiological stimuli such as changes in O_2 tension, also in terms of redox state.

Disulfiram (Antabuse®) is a drug used to treat chronic alcoholism since it induces an extreme adverse reaction when taken in the presence of alcohol by inhibition of acetaldehyde dehydrogenase [49]. It is currently being studied for its antiprotozoal activity, and as a treatment for cocaine dependence, cancer, and HIV [50-53]. Disulfiram was used to study how the thiol to disulfide balance changes in the different cell lines in response to a thiol reactant. This drug resulted to be a good agent to characterize the susceptibility of GSH and P-SH to oxidation in cells. We were aware that disulfiram is not a physiological agonist of *S*-glutathionylation, although, being a marketed drug, it can be considered to be of more interest than other chemicals largely used to study this kind of protein posttranslational modification, such as diamide or tert-butyl hydroperoxide. Therefore, some endogenous oxidants were also tested in pilot studies with some cell lines (e.g. BAEC, see Fig 3), but higher concentrations with respect to disulfiram were necessary in order to influence cellular thiol/disulfide levels. Additionally, since it is known that

inflammatory stimuli, such as those evoked by lipopolysaccharide, or by interleukins and cytokines, induce *S*-glutathionylation in site-specific protein targets [54,55], we also treated BAEC with 100 U/ml tumor necrosis factor α (TNF-α) for 1 h at 37°C. Under these conditions, TNF-α is known to stimulate reactive oxygen species production, probably by activation of NADPH oxidase [56]. Nevertheless, we could not find any significant increase of PSSG by applying our procedure (not shown). It is possible that by these signaling pathways only some specific proteins are targeted and that the consequent increase in PSSG is masked by background levels of already existing *S*-glutathionylated proteins. Here, more qualitative methods are probably needed, in order to assess the site-specific *S*-glutathionylation [10, 33-35].

Disulfiram has the characteristic of being able to react both with GSH and with P-SH, depending on the reactivity and the concentration of the –SH group. The reaction with GSH promotes the formation of GSSG (thus decreasing the GSH to GSSG ratio) and, in some cases, the S-glutathionylation by thiol/disulfide axchange reactions. Instead, by reaction with P-SH, PSSG can form without the GSSG increase [57]. The rise of GSSG and the decrease of the ratio GSH/GSSG was observed in all the cells studied, although at different extents, at the highest concentration of disulfiram (Fig. 3 B,5 A, Table 3). In contrast, PSSG levels were invariably increased by any concentration of disulfiram in most cells (Fig. 3 C). It is noteworthy that GSH/PSSG and GSSG/PSSG ratio decreased even at low doses of the drug in almost all analyzed cell lines (Fig. 4 B, C, Table 3), suggesting that PSSG represents a biomarker of oxidative stress more sensitive than GSH and GSSG when the cells are exposed to thiol specific oxidants. Hence, the GSH/PSSG ratio resulted markedly more sensitive than the GSH/GSSG ratio (Table 3) to low doses of the thiol reactant and showed a concentration-dependence in almost all the cells studied. In conclusion, our study proposes a new and easy procedure to measure GSH and its related oxidized forms in cell cultures, which avoids artificial oxidation during the pre-analytical steps. By application of this procedure, the GSH/GSSG ratio, a widely investigated biomarker of oxidative stress, can be measured in a more accurate way. In addition, our data support the analysis of PSSG as an

additional and reliable biomarker of oxidative stress in cultured cells, as this parameter was shown to be particularly sensitive to low micromolar concentrations of the oxidant agent.

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Figure Legend

Figure 1. Study of the protective effect of NEM. BAEC were treated (black bars) or not (gray bars) with 5 mM NEM. Cells were then lysed by TCA addition and analyzed for the content of GSH, GSSG and PSSG by HPLC. **p<0.01 vs NEM treatment. Data are the mean ± SD, n = 3.

Figure 2. Study of the protective effect of NEM on treated cells with H_2O_2. BAEC were treated with 0.2 mM H_2O_2 for 10 min and then were washed with buffer containing (black bars) or not (light and dark gray bars) 5 mM NEM. Dark gray bars: BCNU added to washing buffer. Cells were then lysed by TCA addition and analyzed for the content of GSH, GSSG and PSSG by HPLC. **p<0.01 vs NEM treatment; §§p<0.01 vs washing buffer without NEM. Data are the mean ± SD, n = 3.

Figure 3. Thiol and disulfide levels in several cell lines treated with disulfiram. Cells were treated with disulfiram (freshly dissolved) in the concentration range 0-200 □M for 15 minutes, stabilized with 5 mM NEM and then lysed by TCA. GSH (panel A) and GSSG (panel B) were measured on the clear supernatant and PSSG (panel C) on the protein pellets. Data are the mean of three replicated experiments. SD for some data was omitted for graphical reasons. *p<0.05 vs 0 □M disulfiram; **p<0.01 vs 0 □M disulfiram.

Figure 4. Thiol and disulfide ratios in several cell lines treated with disulfiram. GSH/GSSG (panel A), GSH/PSSG (panel B) and GSSG/PSSG (panel C) ratio was calculated from data shown in Figure 4. Data are the mean of three replicated experiments. SD for some data was omitted for graphical reasons. **p<0.01 vs 0 □M disulfiram.

Table 1. Recovery of GSH, GSSG, PSSG added to BAEC pre-treated with NEM. BAEC were washed twice with PBS containing 5 mM NEM, after culture medium removal. Cells were then lysed by TCA treatment and collected after scraping. Standard solutions of GSH, GSSG and actinSSG were added to cells immediately before TCA treatment. Data are the mean ± SD. Data in brackets indicate the mean recovery.

Sample	**GSH nmol/mg prot**	**GSSG pmol/mg prot**	**PSSG pmol/mg prot**
BAEC	35.3±4.7	217±27	110±15
BAEC +GSH 30 nmol/mg prot	69.7±9.2 (107%)		
BAEC + GSH 60 nmol/mg prot	94.0±11.3 (98%)		
BAEC + GSSG 200 pmol/mg prot		391±53 (94%)	
BAEC + GSSG 300 pmol/mg prot		559±72 (108%)	
BAEC + GSSG 400 pmol/mg prot		593±22 (96%)	
BAEC + actinSSG 100 pmol/mg prot			214±32 (102%)
BAEC +actinSSG 500 pmol/prot			596±85 (98%)
BAEC + actinSSG 1 nmol/mg prot			1021±76 (109%)

Table 2. Basal levels of GSH, GSSG, PSSG in several cell lines. Cells were washed twice with PBS containing 5 mM NEM after culture medium removal. Cells were then lysed by addition of TCA and collected after scraping. GSH, GSSG and PSSG were measured by HPLC. Data are the mean ± SD, n=3

Cell lines	GSH nmol/mg prot	GSSG pmol/mg prot	PSSG pmol/mg prot	GSH/ GSSG	GSH/ PSSG	GSSG/ PSSG
BAEC	33.9±5.5	217±16	110±17	156	308	1.97
HUVEC	50.8±8.4	137±11	24.3±1.2	372	2116	5.78
Panc-1	48.0±9.2	153±13	87.1±10	313	551	1.75
NT2-D1	34.5±2.7	63.0±8.0	27.8±3.0	545	1277	2.33
A549	106±11.0	293±13	50.1±7.5	361	2120	5.86
RD	91.6±3.2	283±25	107±3	323	856	2.64
HEK293	65.6±12.0	123±17	47.3±3.6	532	1395	2.61
T98G	130±11.2	610±65	330±41	213	393	1.84
HaCaT	39.8±7.6	153±7	20.6±1.4	260	1990	7.65
U87	18.8±0.21	22.0±1.42	140±26	868	134	0.15
IMR90	56.5±5.2	97.0±13.4	28.3±3.7	584	2017	3.46
BRC-230	44.8±6.2	120±11	26.4±2.4	373	1723	4.61
MCF-7	68.8±8.0	237±28	33.9±3.0	291	2084	7.18
A 375	47.2±6.0	207±30	233±13	228	202	0.88
LNCaP	33.6±4.35	127±13	20.7±7.1	265	1680	6.35
LNCaP-Rbic	21.8±1.60	87.0±4.6	14.0±3.3	251	1557	6.21

Table 3. The lowest concentration of disulfiram able to affect the levels of GSH, GSSG and PSSG in several cell lines. Cells were treated with disulfiram in the concentration range 0-200 μM for 15 minutes, stabilized with 5 mM NEM and then lysed by TCA. GSH and GSSG were measured on the clear supernatant, PSSG on the protein pellets. For each cell line the lowest concentration of disulfiram (μM) able to influence significantly the levels of GSH and its disulfide forms with respect to control cells is reported. n=3

Cell lines	GSH	GSSG	PSSG	GSH/ GSSG	GSH/ PSSG	GSSG/ PSSG
BAEC	50	200	10	50	10	10
HUVEC	200	200	10	200	10	10
Panc-1	>200	50	10	50	20	10
NT2-D1	>200	200	20	200	10	10
A549	>200	200	20	200	10	10
RD	200	50	20	50	10	10
HEK293	50	20	10	20	10	10
T98G	200	200	200	200	200	50
HaCaT	>200	200	10	200	10	10
U87	200	200	20	200	50	50
IMR90	200	50	10	50	10	10
BRC-230	>200	200	10	200	10	10
MCF-7	>200	200	10	200	10	10
A 375	10	10	10	10	10	10
LNCaP	>200	200	20	200	10	10
LNCaP-Rbic	>200	200	20	200	10	10

Table 1S. Basal levels of GSH, GSSG, PSSG in several cell lines cultured at 3% O_2. Cells were washed twice with PBS containing 5 mM NEM after culture medium removal, lysed by addition of TCA and collected after scraping. GSH, GSSG and PSSG were measured by HPLC. Data are the mean ± SD, n=3. Data in brackets refer to values in the same cells cultured at atmospheric oxygen concentration (~21%, data are from Table 2). *p<0.05 vs cells cultured at atmospheric oxygen concentration.

Cell lines	GSH nmol/mg prot	GSSG pmol/mg prot	PSSG pmol/mg prot	GSH/ GSSG	GSH/ PSSG	GSSG/ PSSG
NT2-D1	47.6±4.75*	88.9±9.9*	33.9±2.07	535	1404	2.62
	(34.5±2.7)	(63.0±8.0)	(27.8±3.0)	(545)	(1277)	(2.33)
A549	120±11.4	253±47	45.6±2.9	474	2631	5.55
	(106±11.0)	(293±13)	(50.1±7.5)	(361)	(2120)	(5.86)
T98G	92.8±14.1*	597±35	293±26	155	317	2.02
	(130±11.2)	(610±65)	(330±41)	(213)	(393)	(1.84)

Supplementary Methods

Study of the thiol masking effect of N-ethylmaleimide (NEM) on BAEC

At confluence, the culture medium was removed and BAEC were washed twice (1 min each) with phosphate buffered saline solution, pH 7.4, containing NEM at different concentrations: 0, 0.25, 0.5, 1, 2 mM. Cells were then lysed by treatment with 0.5 ml of 4 % (w/v) trichloroacetic acid (TCA) and collected after scraping. Analysis of free glutathione (GSH) in the acidified supernatant was performed by HPLC after labeling with the fluorescent probe monobromobimane (mBrB) [1]. Briefly, 0.1 ml supernatant was reacted with 0.5 mM mBrB (final concentration) at about pH 8.0 (obtained by addition of 0.02 ml of 2 M Tris base) for 10 minutes at room temperature in the dark. Samples were then acidified by treatment with 5 □l of 1M HCl and analyzed by HPLC.

Study of the appropriate concentration of dithiothreitol (DTT)

Glutathione disulfide (GSSG) and S-glutathionylated proteins (PSSG) were measured by quantifying by HPLC the GSH released by the cleavage of the disulfide bridge by DTT and labeled with mBrB [2]. Briefly, for GSSG analysis, one aliquot of supernatant was reacted with 3 volumes of dichloromethane for 10 minutes on a rotator shaker in order to remove the excess NEM. One hundred microliters of supernatant were then treated with 0-0.8 mM DTT (final concentration range, 5 mM stock solution) in the presence of 20 □l of 2 M Tris base. After a 20-minutes incubation at room temperature, samples were deproteinized by addition of 20 □l of 60% (w/v) TCA and centrifugation at 10,000xg for 2 minutes. One hundred microliters of supernatant were then reacted with 3 □l of 40 mM mBrB in the presence of 20 □l of 2 M Tris base for 10 minutes at room temperature in the dark. Finally, samples were acidified by addition of 5 □l of 37% (v/v) HCl and analyzed by HPLC.

For PSSG analysis, protein pellets were washed twice with 1.5% (w/v) TCA and resuspended in 0.3 ml of 0-0.8 mM DTT (final concentration range, 5 mM stock solution), 1 mM K_3EDTA and 15 □l of 2 M Tris base. Samples were incubated for 20 minutes at room temperature

on a rotator shaker and then deproteinized by addition of 30 □l of 60% (w/v) TCA and centrifugation at 10,000xg for 2 minutes. One hundred microliters of supernatant were then reacted with 4 □l of 40 mM mBrB and 20 □l of 2 M Tris base for 10 minutes in the dark. Finally, samples were acidified by addition of 5 □l of 37% (v/v) HCl and analysed by HPLC.

Cell cultures

A549 (human lung carcinoma-derived cells), NT2-D1 (human pluripotent embryonal carcinoma-derived cells), and T98G (human brain glioblastoma-derived cells) were seeded and grown for 48 hours in a humidified atmosphere of 92% N2, 3% O2 and 5% CO2 at 37°. The culture medium was removed and cells were washed twice (1 min each) with phosphate buffered saline solution pH 7.4 (PBS) containing 5 mM NEM (Sigma Aldrich, Milan, Italy). Cells were then lysed by treatment with 0.5 ml of 4 % (w/v) trichloroacetic acid (TCA, Sigma Aldrich, Milan, Italy) and collected after scraping. Samples were immediately analyzed.

Figure Legends

Figure 1 Supplementary. Study of the alkylating effect of NEM. BAEC were treated with 0-2 mM NEM after medium removal, then lysed by TCA addition and analyzed for the content of free GSH by HPLC. Data are the mean ± SD, n= 3.

Figure 2 Supplementary. Study of the reducing effect of dithiotreitol. BAEC were treated with 5 mM NEM after medium removal, then lysed by TCA addition and analyzed for the content of GSSG (black circles) and PSSG (white circles) using different concentrations (0-0.8 mM) of DTT by HPLC. Data are the mean ± SD, n = 3

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Figure 1

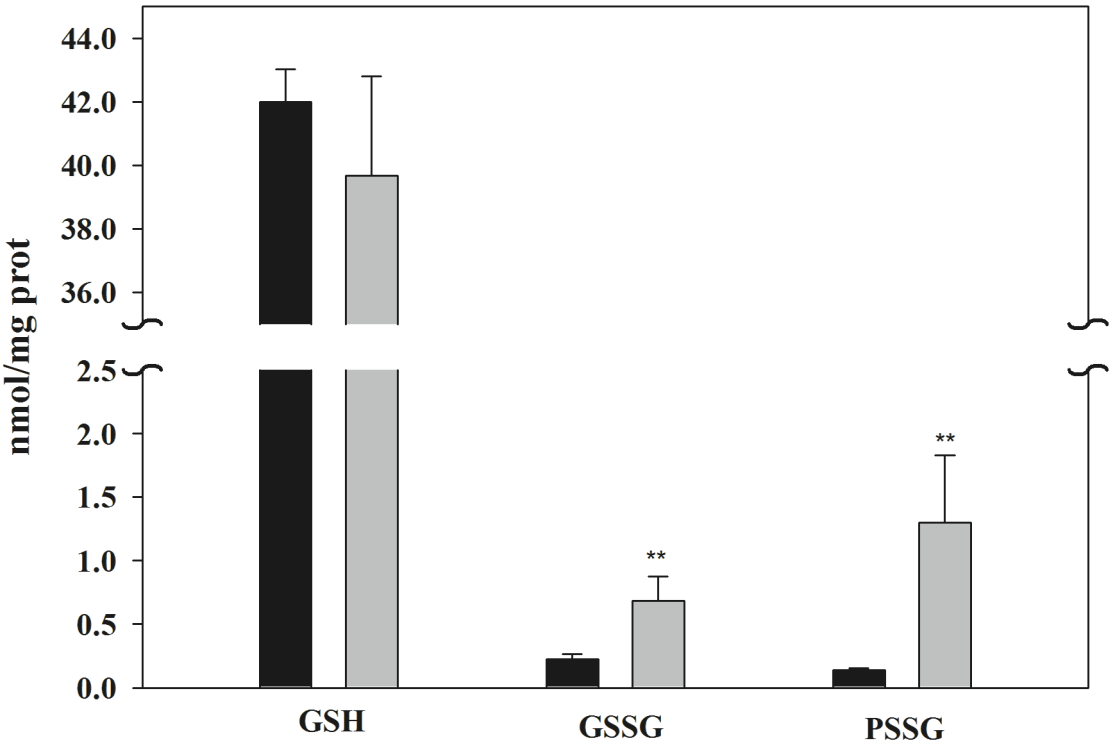

Figure 2

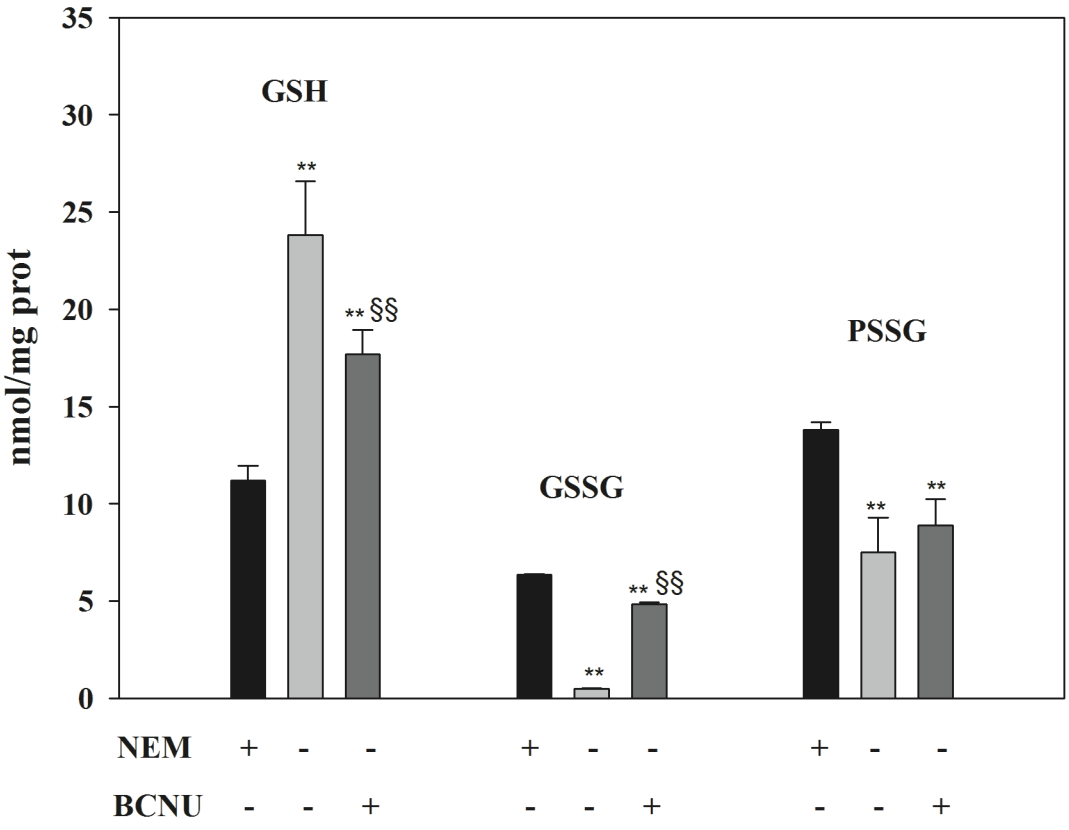

Figure 3A

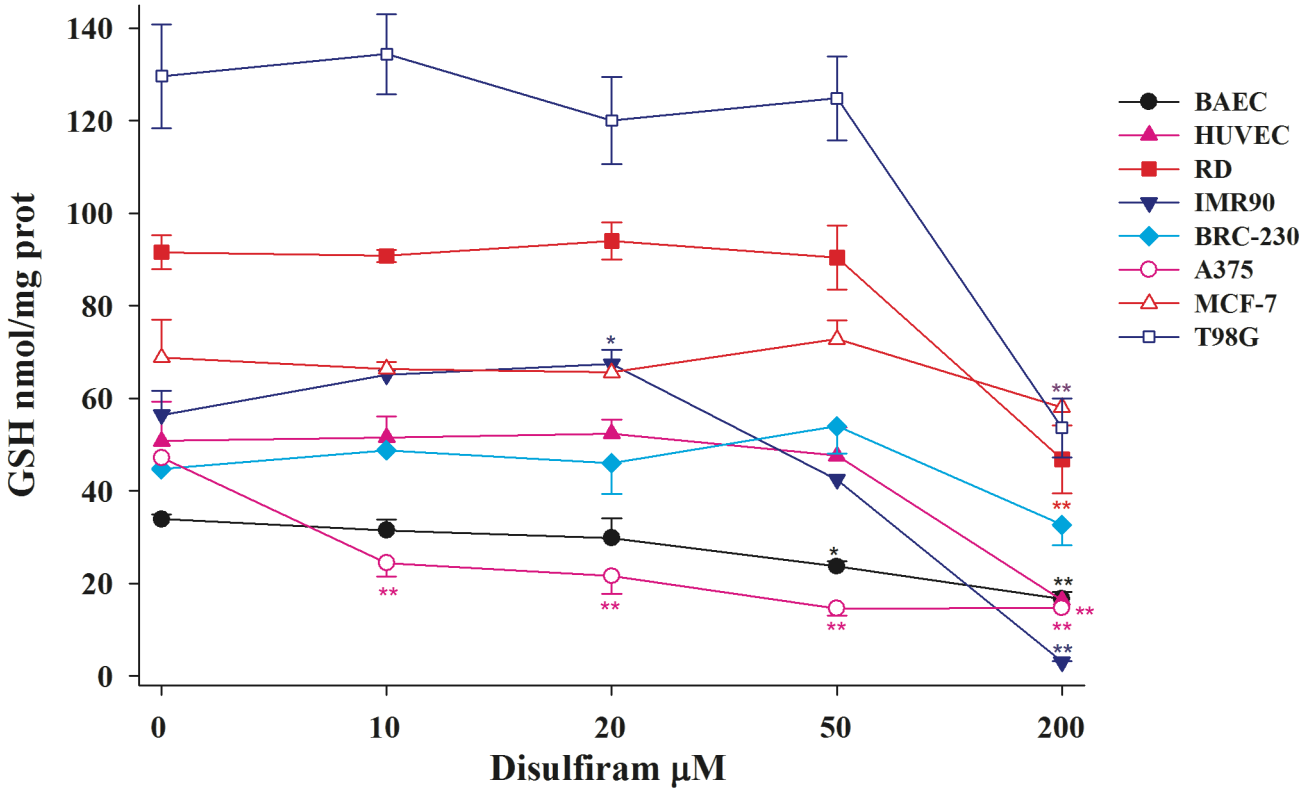
GSH nmol/mg prot
140
120
100
80
60
40
20
0
0
10
20
50
200
Disulfiram µM
BAEC
HUVEC
RD
IMR90
BRC-230
A375
MCF-7
T98G

Figure 3B

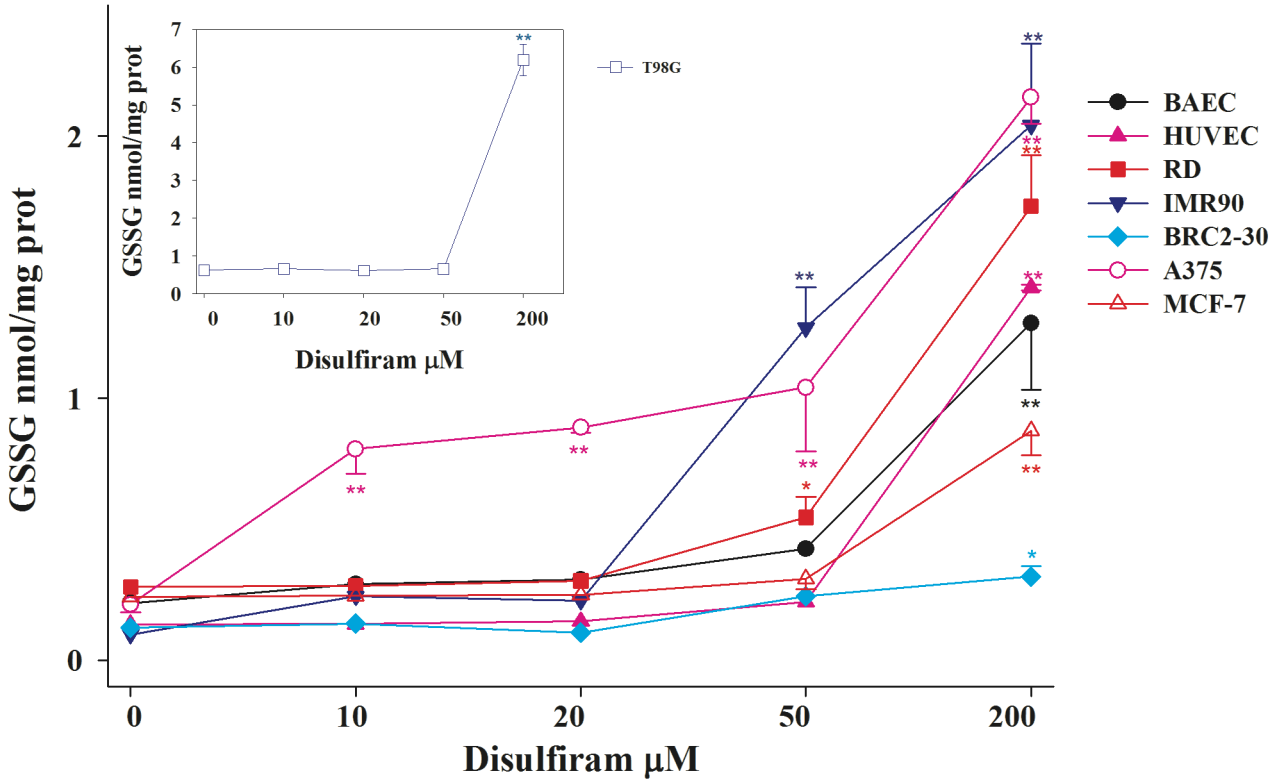
GSSG nmol/mg prot
Disulfiram µM
0
10
20
50
200
1
2
BAEC
HUVEC
RD
IMR90
BRC2-30
A375
MCF-7
T98G
GSSG nmol/mg prot
Disulfiram µM
0
1
2
3
4
5
6
7

Figure 3C

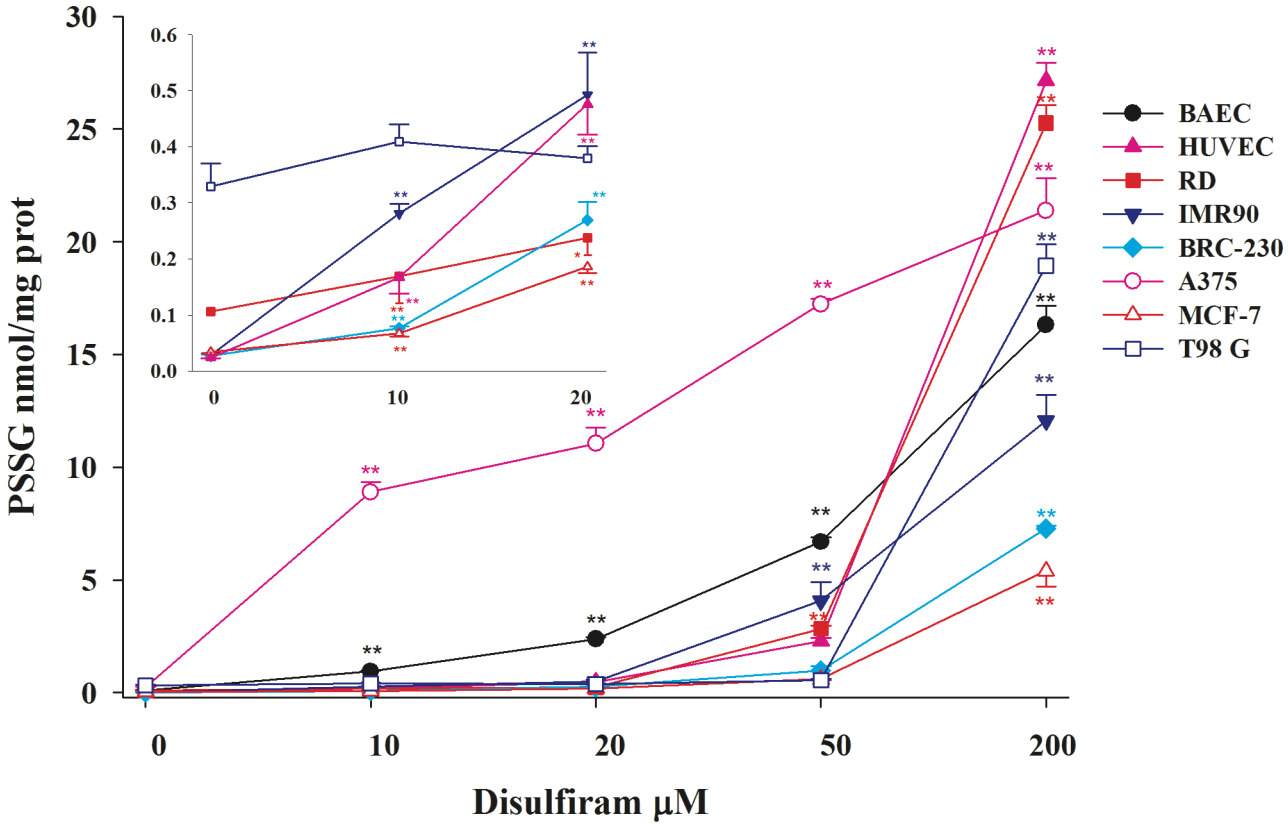
PSSG nmol/mg prot
Disulfiram µM
0
10
20
50
200
BAEC
HUVEC
RD
IMR90
BRC-230
A375
MCF-7
T98 G

Figure 4A

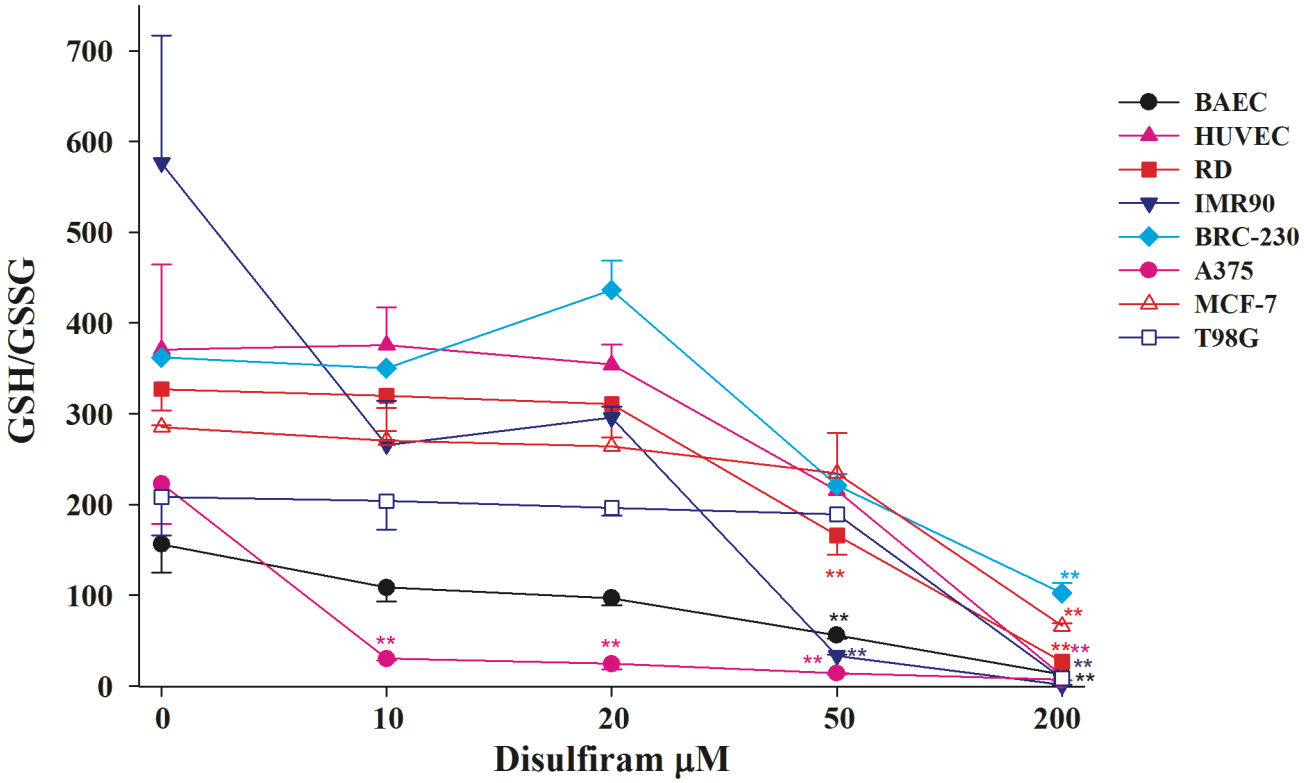
GSH/GSSG
700
600
500
400
300
200
100
0
0
10
20
50
200
Disulfiram μM
BAEC
HUVEC
RD
IMR90
BRC-230
A375
MCF-7
T98G

Figure 4B

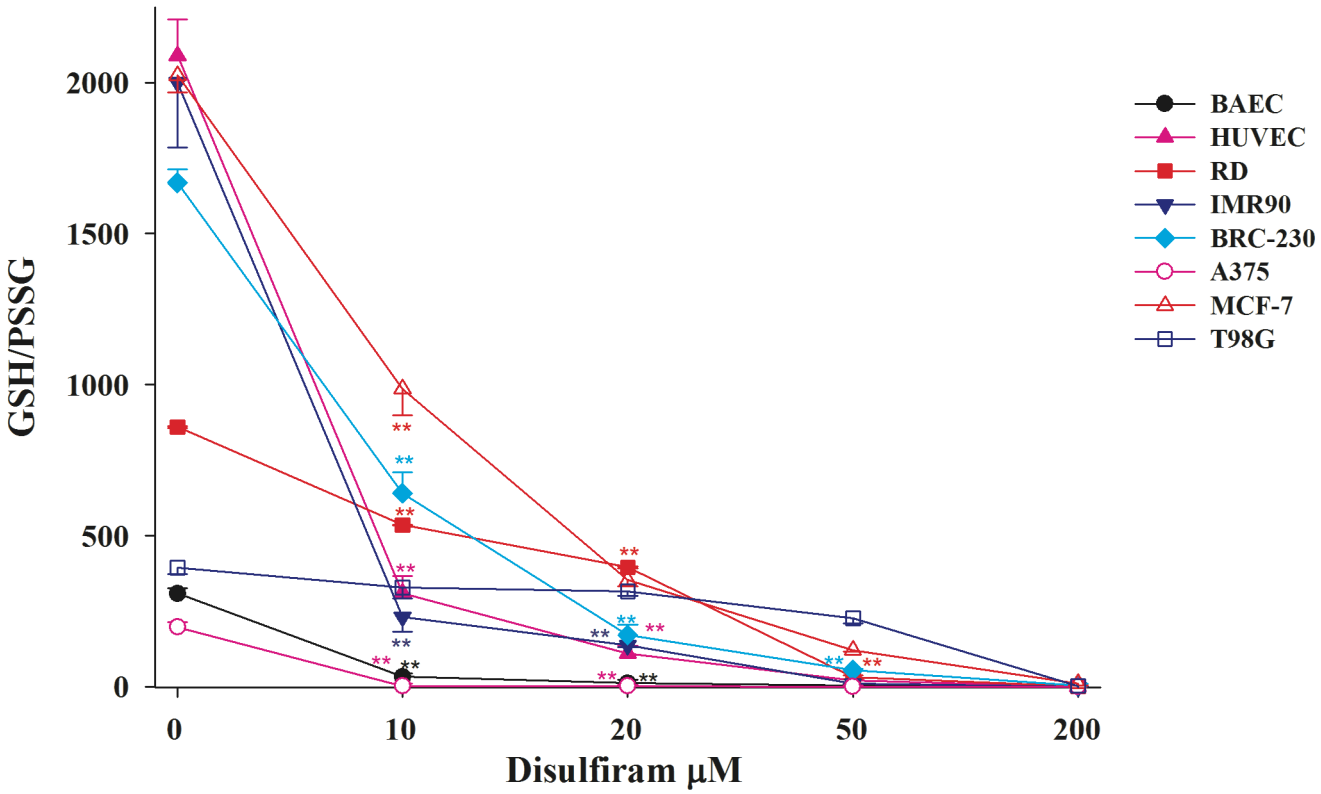
GSH/PSSG
2000
1500
1000
500
0
0
10
20
50
200
Disulfiram μM
BAEC
HUVEC
RD
IMR90
BRC-230
A375
MCF-7
T98G

Figure 4C

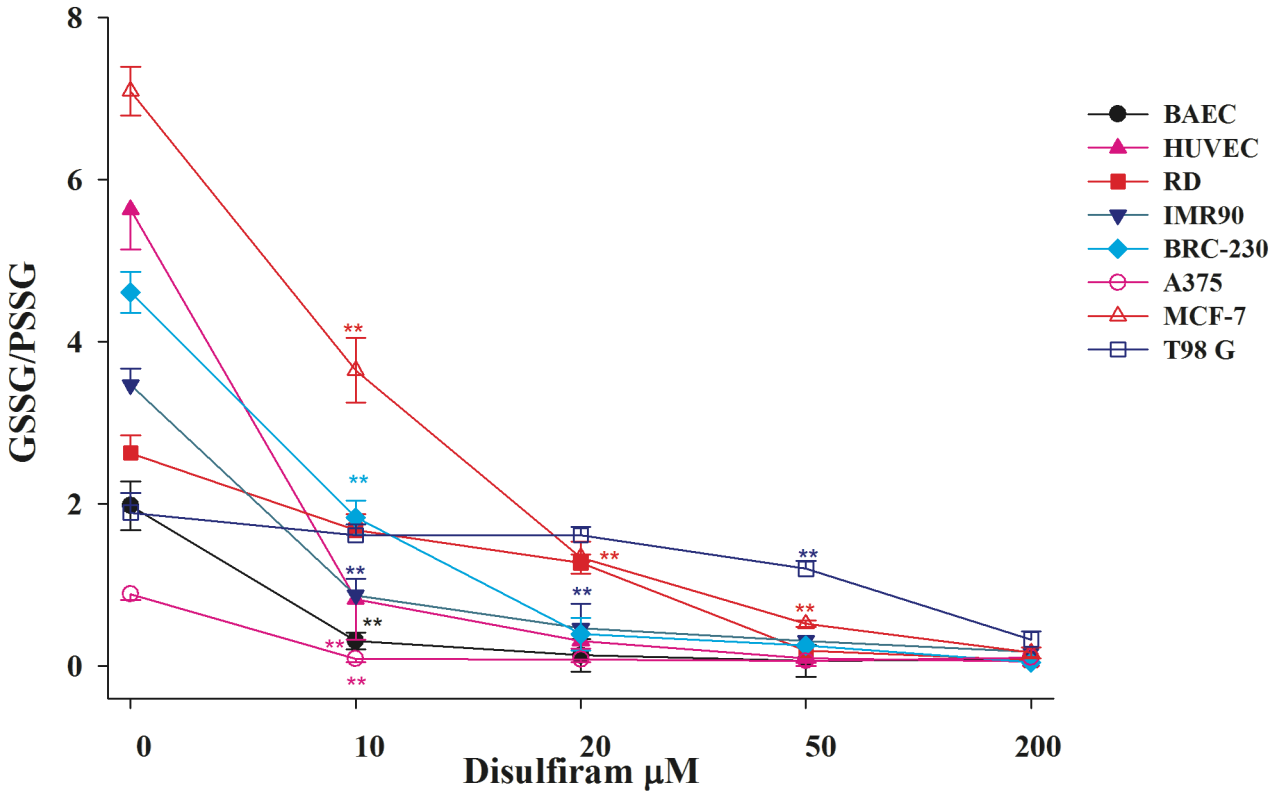
GSSG/PSSG
8
6
4
2
0
0
10
20
50
200
Disulfiram µM
BAEC
HUVEC
RD
IMR90
BRC-230
A375
MCF-7
T98 G

Figure 1S

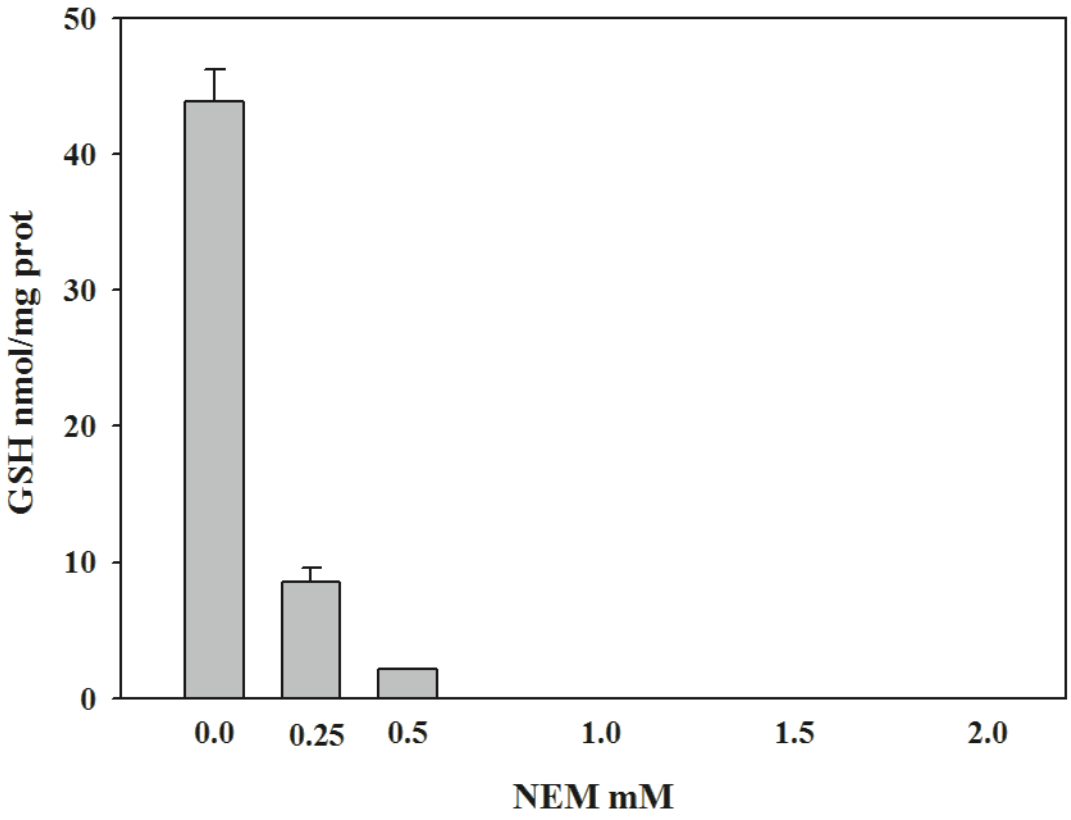

Figure 2 Supplementary